Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

Multi-Walled Carbon Tubes (synthetic graphite in tubular shape) with a geometric tube diameter range ≥ 30 nm to < 3 µm and a length ≥ 5 µm and aspect ratio ≥ 3:1, including Multi-Walled Carbon Nanotubes, MWC(N)T

EC Number: -

CAS Number: -

Index Number: tba

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CONTENTS

1 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

1.1.1 [Study 1]

Study 1 reference:

Kasai et al. (2015) : Thirteen-week study of toxicity of fiber-like multi-walled carbon nanotubes with whole-body inhalation exposure in rats. Nanotoxicology 1-10.

Test type

Inhalation

Similar to OECD Guideline TG413

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity:* > 99.5%
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

Material and methods

Test material: MWNT-7 (Hodogaya, Lot No. 071223, 080126); Mean D: 90.7 nm; Mean L: 5.7 μm (48.7% > 5 μm)
MMAD = 1.4-1.6 μm (~80% mass as inhalable fraction)

Whole-body exposure to 0.2, 1 or 5 mg/m3 cyclone-sieve generated MWCNT aerosol for 6 h/day, 5 days/week for 13 weeks;
Doses corresponded to number concentrations of about 115000, 5770000, and 2933000 cpm (i. e. “particles” > 0.3 μm)
rat /F344 DuCrlCrlj/ m/f (n=10/group)

Results

Lung burden increased dose-dependently: 3.23, 21.2 and 120.3 µg/left lung in males (lung burden in females was ca. 1.5 times lower because of physiologically lower breathing rate). The total lung burden was calculated to be 54-81% of the simulated lung burden estimated by the Multiple-Path Particle Dosimetry (MPPD) model.

MWCNT in the lung were primarily detected within alveolar macrophages but few single non-phagocytosed fibres were also found in the bronchiolar and alveolar spaces. Notably, two types of alveolar macrophages were predominant in the alveoli: alveolar macrophages which phagocytosed many MWCNTs and alveolar macrophages with foamy cytoplasm which phagocytosed only a few long MWCNT fibres.

MWCNT deposition in bronchus-associated lymphoid tissue (BALT) and mediastinal lymph nodes was found in all MWCNT-exposed rats. In the highest dose group, occasionally MWCNT could be observed in the visceral subpleural areas and in the parietal pleura at the diaphragm (other organs were not explored).

MWCNTs were also deposited in the nasal cavity (respiratory epithelium) of all MWCNT-exposed rats, primarily in non-ciliated respiratory epithelia (mucosa) and the lamina propria of the nasal cavity.

MWCNT deposition and retention increased in a concentration- and time-dependent manner.

1.1.2 [Study 2]

Study 2 reference:

Xu et al. (2014) Size- and shape-dependent pleural translocation, deposition, fibrogenesis, and mesothelial proliferation by multiwalled carbon nanotubes. Cancer Science 105 (7); 763-769.

Test type

Transtracheal intrapulmonary spraying

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* 1. MWCNT-L
  2. MWCNT-S
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

1. MWCNT-L; D: 150 nm; L. 8 µm ; Mean length: 7.34 µm (needle-shaped)

2. MWCNT-S: D: 15 nm; L: 3 µm (cotton candy like aggregates)

Exposure to 1.625 mg MWCNT/rat (total amount) over a period of 24 weeks

Rat/F344/m (n=6/group)

Results

The longer, needle-like (MWCNT-L) but not the shorter, "cotton candy"-type (MWCNT-S) MWCNT translocated into the pleural cavity and deposited primarily at the parietal pleura.

MWCNT-S were found phagocytosed in alveolar macrophages close to the visceral pleura.

MWCNT-L were found in extrapulmonary organs, such as lymph nodes (mediastinal, submandibular and mesentery), few tubes were also in the liver, kidney, spleen and brain. MWCNT-S were not detected in extrapulmonary organs.

1.1.3 [Study 3]

Study 3 reference:

Xu et al. (2012): Multi-walled carbon nanotubes translocate into the pleural cavity and induce visceral mesothelial proliferation in rats. Cancer Science 103 (12); 2045-2050.

Test type

Transtracheal intrapulmonary spraying

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* 1. MWCNT-M
  2. MWCNT-N
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

1. MWCNT-M (= MWNT-7, Mitsui): Median length: 4.47 µm; Average length: 5.11 µm; 3.82 x 1011 fibres/g

2. MWCNT-N (Nikkiso): Median length: 3.02 µm; Average length: 3.64 µm; 3.46 x 1011 fibres/g

Positive control: Crocidolite (1.25 mg/rat)

Exposure to 1.25 mg MWCNT/rat (total amount) over a period of 9 days

Rat/F344/m (n=6/group)

Results

Like crocidolite, both MWCNT types translocated into the pleural cavity when administered into the rat lung. Here they were mainly found within pleural macrophages. Only few MWCNT and crocidolite fibres were observed penetrating through the visceral pleura.

In addition, MWCNT and crocidolite frequently deposited in the mediastinal lymph nodes (mostly found phagocytosed by macrophages).

Few fibres were also detected in liver sinusoid cells, blood vessel wall cells in the brain, renal tubular cells and spleen sinus and macrophages without reporting how they could get there.

1.1.4 [Study 4]

Study 4 reference:

Aiso et al. (2011): Translocation of Intratracheally Instilled Multiwall Carbon Nanotubes to Lung-Associated Lymph Nodes in Rats. Industrial Health 49 (2); 215-220.

Test type

Intratracheal instillation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*

- *Batch number*
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWNT-7 (Mitsui): Fibres of 35.5 or 53 nm diameter

Exposure to 0, 40 or 160 μg/rat

Rat/F344/DuCrlCrlj/m (n=8/group, m)

Rats were sacrificed on day 1, 7, 28 or 91 following instillation

Results

MWCNT migrated to the right and left posterior mediastinal lymph nodes and – to a lesser extent – to the parathymic lymph node.

The deposition of MWCNT in these lymph nodes increased gradually and dose-dependently during the postexposure period.

At 91 days postexposure, aggregated nodal macrophages laden with MWCNT were observed, which the authors speculated to progress into microgranulomas.

1.1.5 [Study 5]

Study 5 reference:

Czarny et al. (2014): Carbon nanotube translocation to distant organs after pulmonary exposure: insights from in situ (14)C-radiolabeling and tissue radioimaging. ACS Nano 8 (6); 5715-24.

Test type

Pharyngeal aspiration

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* Synthesised ^{14}C-MWCNT
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*

- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWCNT: L: 500 nm – 12 µm (mean: 3.9 µm); D: 10-150 nm (mean: 40 nm)

Single dose exposure (20 µg) of pristine 14C-MWCNT (285 x 103 Bq) (n=4)

Analysis 1 and 7 days, 1, 3, 6, 9 and 12 months after lung exposure.

Mice/BalB/c/f

Results

Translocation of MWCNT to distant organs after pulmonary exposure investigated by using in situ radiolabelling, combining tissue radioimaging of organ tissue sections to ex vivo analysis of MWCNTs by electron microscopy.

10% of the calculated applied dose in the lung (10 µg) was retained in the lung after 12 months.

Whereas most of the deposited dose was assumed to be cleared early via the mucociliary escalator, concomitant re-location to distant organs was observed. In particular 200 ng MWCNT in the spleen and in liver 75 ng MWCNT was detected after 12 months postexposure. Little radioactivity was detected in the heart and none in brain and thymus after 360 days.

TEM analysis detected MWCNT of different lengths and diameters in peripheral organs such as spleen and bone marrow (MWCNT of 40 nm diameter in spleen, one 4 µm long fibre was found in liver extracts), accumulating over the whole period of the study, from day 1 to month 12, without decreasing, indicating high biopersistence.

1.1.6 [Study 6]

Study 6 reference:

Mercer et al. (2013a)*:* Extrapulmonary transport of MWCNT following inhalation exposure. Particle and Fibre Toxicology 10

Test type

Inhalation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number:* Lot No. 061220-31
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWNT-7 (Hodogaya): L: 4.3 µm (mean); Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm.

1.32% metal contamination (1.06% iron)

Whole-body exposure of 5 mg/m^3 for 12 days

Mice/C57BL/6J/m (n= 7-9/group)

Postexposure: 1, 14, 84, 168, and 336 days

Results

The lung burden and changes in the compartmentalisation of MWCNT within the lung during almost one year of postexposure (p.e.) was investigated.

By using sensitive optical techniques, MWCNT could be detected in liver, kidney, heart, and brain already 1 day p.e. Opposed to the initial lung burden mainly consisting of agglomerates, MWCNT found in extracellular organs were individual tubes ('singlets') of about 7-8 µm in length or small assembled structures containing few tubes.

These accumulated over the 336-day follow-up period. In the tracheobronchial lymph nodes, which contained the highest share of translocated MWCNT, the initial presence of singlets disappeared in favour of multiple fibre-containing foci by day 336 p.e.

Expressed as percentage of deposited lung burden (28.1 µg/lung), tracheobronchial lymph nodes contained 1.08 and 7.34% at day 1 and day 336 p.e., respectively.

The percentages in liver (the target organ containing second-highest amounts of MWCNT) were 0.002 and 0.027% 1 and 336 days p.e., respectively.

In general, the increase over time in extrapulmonary organs was 6-7-fold (excluding the chest wall, where MWCNT burden did not change significantly). The authors assumed that the tracheobronchial lymphatic is a major route for systemic delivery of inhaled MWCNT.

1.1.7 [Study 7]

Study 7 reference:

Mercer et al. (2013): Distribution and fibrotic response following inhalation exposure to multi-walled carbon nanotubes. Particle and Fibre Toxicology 10

Test type

Inhalation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities:* 1.32% metal contamination (1.06% iron)
- *Batch number:* Lot No. 061220-31
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWNT-7 (Hodogaya): L: 4.3 µm (mean); Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm

Whole-body exposure of 5 mg/m3) for 12 days

Mice/C57BL/6J/ m (n= 7-9/group)

Postexposure: 1, 14, 84, 168, and 336 days

Results

Total lung burdens successively but incompletely decreased from 28 to 18 µg from day 1 to day 336 postexposure.

Initially, 84% of the total lung burden was found in the alveolar region (including 1.2% in subpleural tissue), 16% in the airways. Clearance reduced the alveolar macrophage burden of MWCNTs by 35 percent between 1 and 168 days post-exposure, while the content of MWCNTs in the alveolar tissue increased by 63 percent. At 336 days, 95.8% of the initial lung burden remained in the alveolar region (including 4.8% in subpleural tissue), whereas 4.2% was found in the airways.

Within the alveolar region, the burden contained in alveolar macrophages was initially 3-fold that of the alveolar tissue, including subpleural alveolar tissue (56% vs. 20%). However, over time it declined due to clearance by 35% between 1 and 336 days postexposure, while the alveolar tissue burden increased by 36%.

Large MWCNT structures containing greater than 4 fibres were 53.6% of the initial lung burden and accounted for the majority of the decline with clearance, while lung burden of singlet MWCNT was essentially unchanged

1.1.8 [Study 8]

Study 8 reference:

Porter et al. (2013): Acute pulmonary dose-responses to inhaled multi-walled carbon nanotubes. Nanotoxicology 7 (7); 1179-1194.

Test type

Inhalation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities:* 1.32% metal contamination (1.06% iron)
- *Batch number:* Lot No. 061220-31
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWNT-7 (Hodogaya): Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode):

MMAD = 1.5 µm

Whole-body exposure of 10 mg/m^3 for 2, 4, 8, or 12 days

Mice/C57BL/6J/ m (n= 7-9/group)

Results

After 4 days of exposure, 76% of CNT lung burden was localised in the alveolar region, 14.6% of which was distributed in the airspace, 11.9% in the alveolar (including subpleural) tissue and 49.2% in alveolar macrophages.

MWCNT were also found in tracheobronchiolar lymphocytes and were shown to reach the pleural wall, which they occasionally penetrated.

Lung burden was calculated to be equivalent to humans in occupational settings.

1.1.9 [Study 9]

Study 9 reference:

Murphy et al. (2011)*:* Length-dependent retention of carbon nanotubes in the pleural space of mice initiates sustained inflammation and progressive fibrosis on the parietal pleura. The American Journal of Pathology 178 (6); 2587-2600.

Test type

Intrapleural injection

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWCNT
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

Short straight MWCNT: D: 20-30 nm, L: 0.5-2 μm [Nanostructured & Amorphous Materials, Inc.]

"Length controls": - Nickel nanowires of 200 nm diameter as short (4.3 μm) and long (24 μm) fractions

Analysis 1-24h after injection by Single-Photon Emission Computed Tomography (SPECT/CT) imaging

single dose of 5 ^{111}In-radiolabeled MWCNT μg/mouse

Mice (n= 4-5/group)

Results

Indirect evidence was presented that passage through parietal stoma to mediastinal lymph nodes is dependent on MWCNT length. Thus short fibres are able to be cleared via the lymphatics, whereas long fibres are retained at the parietal pleura. The length-dependent pleura passage block was confirmed by nickel wire administration of defined lengths.

1.1.10 [Study 10]

Study 10 reference:

Porter et al. (2010*):* Mouse pulmonary dose- and time course-responses induced by exposure to multi-walled carbon nanotubes. Toxicology 269 (2-3); 136-147.

Test type

Pharyngeal aspiration

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities:* 0.78% metal contamination (0.31% iron)
- *Batch number:* Lot No. 05072001K28

- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWNT-7 (Mitsui): D: 49 nm; L: 3.86 µm (median)

Single dose exposures (10, 20, 40 or 80µg)

Examinations 1, 7, 28, or 56 days (d) post-exposure (p. e.).

Mice/C57BL/6J/ m (n=4/group)

Results

The aspirated dose was widely distributed throughout the lungs and rapidly incorporated into the alveolar walls and alveolar cells.

Within one hour after aspiration, fibres were found engulfed by type II alveolar epithelial cells and alveolar macrophages. At later time points MWCNT were generally no longer present on the surface of epithelial cells but had been transported within the alveolar interstitium and/or interstitial cells as well as within macrophages in the interstitium (as single fibres up to larger clusters). Incomplete phagocytosis by macrophages, lasting for weeks, was also observed.

1.1.11 [Study 11]

Study 11 reference:

Mercer et al. (2010): Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes. Particle Fibre Toxicol 7

Test type

Pharyngeal aspiration

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*

- *Impurities:* 0.78% metal contamination (0.31% iron)
- *Batch number:* Lot No. 05072001K28
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWNT-7(Mitsui): D: 49 nm; L: 3.86 µm (median)

Single dose exposures (10, 20, 40 or 80µg)

Examinations 1, 7, 28, or 56 days (d) post-exposure (p. e.).

Mice/C57BL/6J/ m (n=4/group)

Results

At 1 day 18%, 81.6% and 0.6% of the MWCNT lung burden was in the airway, the alveolar, and the subpleural regions, respectively. There was an initial, high density of penetrations into the subpleural tissue and the intrapleural space one day following aspiration which appeared to decrease due to clearance by alveolar macrophages and/or lymphatics by day 7. However, the density of penetrations increased to steady state levels in the subpleural tissue and intrapleural from day 28 - 56.

56 days after exposure to 80 µg MWCNT, it became evident that MWCNT can reach the pleura, as fibres were detected in the subpleural lymphatics. 1 in every 400 fibre penetrations was found either in the subpleural tissue (i.e. the alveolar epithelium adjacent to the pleura) or in the intrapleural space, defined as visceral pleural surface.

1.1.12 [Study 12]

Study 12 reference:

Ryman-Rasmussen et al. (2009): Inhaled carbon nanotubes reach the subpleural tissue in mice. Nature Nanotechnology 4 (11); 747-751.

Test type

Inhalation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWCNT (Helix Material Solutions)
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*: > 94% (mixture of agglomerated and individual nanotubes)
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*
- *Physicochemical properties that may be important when assessing toxicokinetics*

Detailed study summary and results:

[Please provide the test material identity, a detailed study and results transparently and objectively as in the original data source without subjective interpretations.]

Material and methods

MWCNT (Helix Material Solutions): L: 0.3-50 µm; D: 30-50 nm (average)

Nose only exposure to 1 or 30 mg/m3 for 6 h

Mice were sacrificed 1 day, 2 weeks, 6 weeks or 14 weeks post-exposure

Mice/C57BL6/ m (n=10/group)

Results

MWCNT were detected in the subpleura one day after inhalation exposure to 30 mg/m3 for 6h.
Mononuclear cell aggregates on the pleural surface increased in number and size after 1 day and nanotube-containing macrophages were observed within these foci.
Most of the inhaled nanotubes appeared to be cleared, though some remained in the subpleural wall for at least 14 weeks.

2 HEALTH HAZARDS

2.1 Carcinogenicity

2.1.1 Animal data

2.1.1.1 [Study 1]

Study reference:

Kasai, T. et al. (2016): Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. Particle and Fibre Toxicology 13(1):53

Detailed study summary and results:

Test type

Long-term study

Similar to OECD Guideline 451/GLP

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*: > 99.6%/>99.8%
- *Impurities (or a note that the impurities do not affect the classification):* Fe: 4,400 ppm; Cr: 48 ppm; Ni: 17 ppm
- *Batch number:* Hodogaya; Lot No. 080126 for 88 weeks, Lot No. 071223 from week 89

Test animals

- *Species/strain/sex:* rat/ F344/DuCrlCrlj/m/f
- *No. of animals per sex per dose:* 50/sex and dose group
- *Age and weight at the study initiation*: 6 weeks

Administration/exposure

- *Route of administration:* Inhalation (aerosol)
- *duration of test/exposure period:* 104 weeks
- *doses/concentration levels, rationale for dose level selection:* 0, 0.02, 0.2, 2 mg/m^3
- *frequency of treatment:*
- *control group and treatment:* No positive control
- *historical control data*
- *post exposure observation period:*
- *vehicle:*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation*
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions:* Dry-aerosol generated and well dispersed MWNT, constant in concentration and separation of individual tubes in exposure chamber over exposure period
- *method of exposure:* whole body
- *analytical verification of test atmosphere concentrations*
- *particle size: Average length: 5.2/5.7 µm, with 45.1/48.7% of tubes > 5µm;*
  Av. diameter: 83.8/90.7 nm.
  Dispersed fibers collected from inhalation chamber
  Av. length: 5.4-5.9 µm; Av. diameter: 92.9-98.2 nm; MMAD (SD): 1.2-1.4 µm (2.6-3.0)
- *type or preparation of particles (for studies with aerosols)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs:* No development of pleural mesothelioma.
  Dose-dependent lung toxicity, such as epithelial hyperplasia, granuloma and focal fibrosis development, accompanied by altered BALF parameters.
- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights:* Increased lung weights from 0.2 mg/m3 in males and at 2 mg/m3 in females. MWNT-7 lung burdens increased with dose and duration of exposure.
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity:* No macroscopic findings in any other organs, including pleura and peritoneum.
- *tumour incidence data by sex, dose and tumour type:* Significantly increased incidence of lung tumours (mainly bronchiolo-alveolar carcinoma, and combined carcinomas and adenomas) at MWNT-7 exposure of males at 0.2 and 2 mg/m3 and at 2 mg/m3 in females.
- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *tumour incidence data by sex, dose and tumour type*
- *mode of action (genotoxic, non-genotoxic)*

- *toxic response data by sex and dose*
- *tumour latency*
- *statistical methods and results (unless already described with specific test results above*

2.1.1.2 [Study 2]

Study reference:

Xu et al. (2012): Multi-walled carbon nanotubes translocate into the pleural cavity and induce visceral mesothelial proliferation in rats. Cancer Science 103 (12); 2045-2050.

Detailed study summary and results:

Test type

Short-term study

No standard guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:*

  1. MWNT-M (= MWNT-7, Mitsui)
  2. MWCNT-N (Nikkiso)
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* rat/ F344/ m
- *No. of animals per sex per dose:* n=6/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration:* inhalation (Transtracheal intrapulmonary spraying)
- *duration of test/exposure period:* 9d
- *doses/concentration levels, rationale for dose level selection:* 1.25 mg MWCNT/rat: 5 x 250 µg
- *frequency of treatment*
- *control group and treatment:* Positive control: Crocidolite (1.35 mg)

- *historical control data*
- *post exposure observation period*
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation*
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions:* Transtracheal intrapulmonary spraying
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size: 1. MWCNT-M ; Average length: 5.11 µm 3.82 x 1011 fibres/g*
  (Diameter according to WPMN report: 88 nm)
  2. MWCNT-N (Nikkiso) Average length: 3.64 µm 3.46 x 1011 fibres/g
  (Diameter according to WPMN report: 48 nm)
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs:* Both MWCNT types translocated to the pleura as evidenced by pleural cavity lavage, predominantly as phagocytosed material in alveolar macrophages.
  Both MWCNT types and crocidolite induced hyperplastic visceral mesothelial proliferation (PCNA immunostaining), associated with inflammatory cell infiltration and inflammation and fibrotic lesions of the pleural tissues.
  It was assumed that inflammatory action by activated macrophages in the pleura rather than the fibres themselves induced mesothelial lesions (conditioned cell culture media of macrophages treated with MWCNT and crocidolite and the supernatants of pleural cavity lavage fluid from the dosed rats increased mesothelial cell proliferation
  in vitro).
- *food/water consumption*
- *ophthalmoscopic examination*

- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings:* MWCNT were not found in the mesothelial proliferative lesions themselves, as evidenced by polarized light microscopy and scanning electron microscopy of H&E-stained histology slides.
- *tumour incidence data by sex, dose and tumour type*
- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *tumour incidence data by sex, dose and tumour type*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency*
- *statistical methods and results (unless already described with specific test results above*

2.1.1.3 [Study 3]

Study reference:

Xu et al. (2014): Size- and shape-dependent pleural translocation, deposition, fibrogenesis, and mesothelial proliferation by multiwalled carbon nanotubes. Cancer Science 105 (7); 763-769.

Detailed study summary and results:

Test type

Medium-term study

No standard guideline followed

Test substance

Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:

1. MWCNT-L; needle-shaped
2. MWCNT-S; cotton candy like aggregates

- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* Rat/ F344/ m
- *No. of animals per sex per dose:* 6/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration:* inhalation (Transtracheal intrapulmonary spraying)
- *duration of test/exposure period:* 24 weeks
- *doses/concentration levels, rationale for dose level selection:* 1.625 mg MWCNT/rat:13 x 250 µg
- *frequency of treatment*
- *control group and treatment:* No positive control
- *historical control data*
- *post exposure observation period*
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation*
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions:* Transtracheal intrapulmonary spraying
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size :* 1. MWCNT-L; D: 150 nm, L. 8 µm (needle-shaped)
  2. MWCNT-S: D: 15 nm; L: 3 µm; Mean length: 7.34 µm
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*

- *clinical signs:* Needle-shaped MWCNT-L, but not cotton candy-like MWCNT-S, translocated into the pleural cavity and induced fibrotic thickening of the parietal and visceral pleura as well proliferation of both the parietal and visceral mesothelium (PCNA).
  Inflammatory pleural lavage parameters indicated that MWCNT-L elicited a stronger inflammatory reaction in the pleural cavity than MWCNT-S.
  MWCNT-S caused higher inflammatory reactions (cell number and cytokine/chemokine levels) and 8-OHdG formation in the lung compared to MWCNT-L.

- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type*
- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *tumour incidence data by sex, dose and tumour type*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency*
- *statistical methods and results (unless already described with specific test results above*

2.1.1.4 [Study 4]

Study reference:

Suzui et al. (2016): Multiwalled carbon nanotubes intratracheally instilled into the rat lung induce development of pleural malignant mesothelioma and lung tumors. Cancer Sci 107 (7); 924-35.

Detailed study summary and results:

Test type

Long-term (observation) study

No standard guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier :* MWCNT-N (Nikkiso), 3 sieve fractions
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* Rat/F344/ m
- *No. of animals per sex per dose:* 20/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration :* inhalation (Transtracheal intrapulmonary spraying)
- *duration of test/exposure period:* 8 administrations over a 2-week period
- *doses/concentration levels, rationale for dose level selection:* 1 mg/rat
- *frequency of treatment*
- *control group and treatment:* Negative controls: no treatment
- *historical control data*
- *post exposure observation period:* up to 109 weeks (animals surviving ≥ 63 weeks were included in the study)
- *vehicle:* 0.5% Pluronic F68
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation*
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions:* Transtracheal intrapulmonary spraying
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size:* 1. "Unfiltered"; L: 4.2 ± 2.9 µm; D: 30-80 nm (93.4%)
  2. "Flow through"; L: 2.6 ± 1.6 µm; D: 30-80 nm (93.4%)
  3. "Retained"; L: > 2.6 µm (not measurable → dense agglomerates); D: 30-80 nm (93.4%)
  Needle or fibre-like appearance; Ion content: 0.004-0.005%

- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs:* MWCNT remaining in the lungs of rats administered unfiltered
  MWCNT-N and the flow-through and retained MWCNT fractions were 25.4, 48.0 and 26.3%, respectively, of the amount measured at week 2 (= 486 ± 44 µg; 426 ± 116 µg; 268 ± 43 µg, respectively).
- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type:* Pleural malignant mesothelioma as well as lung tumours developed. MWCNT remaining in the lungs of rats administered unfiltered
  MWCNT-N and the flow-through and retained MWCNT fractions were 25.4, 48.0 and 26.3%, respectively, of the amount measured at week 2 (= 486 ± 44 µg; 426 ± 116 µg; 268 ± 43 µg, respectively).
  Pleural malignant mesothelioma as well as lung tumours developed.
  The incidence of lung tumours (bronchiolo-alveolar adenoma and carcinoma) (14/38; 36.8%) together was significantly higher than the control group (0/28; 0%) while no significant differences in the incidence of lung tumours or total tumour burden was found among the three groups administered the different MWCNT-N sieve fractions.
  The incidence of malignant mesothelioma in the three
  MWCNT groups of different sieve fractions combined, 6/38 (15.8%), was significantly higher compared to the two control groups combined, 0/28 (0%). The groups administered the unfiltered

and flow-through fractions had incidences of 3 mesothelioma cases each and the group administered the (highly agglomerated) retained fraction did not have any cases of mesothelioma.

The mesotheliomas were localised in the mediastinal space (Cavum mediastinale) and were shown to originate from mesothelial tissue outside of the lung as shown by negative immunostaining for the lung tumour marker thyroid transcription factor-1 (TFF-1).

Tumour incidence in other organs was not significant compared to controls.

- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency*
- *statistical methods and results (unless already described with specific test results above*

2.1.1.5 [Study 5]

Study reference:

Huaux et al. (2016) : Mesothelioma response to carbon nanotubes is associated with an early and selective accumulation of immunosuppressive monocytic cells. Part Fibre Toxicol 13 (1); 46.

Detailed study summary and results:

Test type

Long-term and short-term observation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7 ("CNT-7"; MWCNT-XNRI-7, Mitsui) = NRCWE-006

  A subset ("short CNT-7") of MWNT-7 was produced by grinding. Both types were annealed at 1500°C.

  In addition, two MWCNT types were tested for their immunosuppressiv potential in short-term tests, which proved non-carcinogenic in previous i.p. mesothelioma experiments: "CNT-M (Muller et al, 2009) and CNT-T (tangled, same as "NTtngl" in Nagai et al., 2011).
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*

- *Batch number*

Test animals

- *Species/strain/sex: Rat/ SPF Wistar rat/ male; Mouse/ C57BL/6/ no data*
- *No. of animals per sex per dose:*

  Rat: n=50 (long-term) or n= 4-5 (short-term) test

  Mouse: Group size as well as study duration for long term experiment not given. for short-term experiment: n= 4-5
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration:* Intraperitoneal injection
- *duration of test/exposure period*
- *doses/concentration levels, rationale for dose level selection*

  Long-term experiment:

  Rat: Single dose of 6 mg of CNT-7 (2x109 WHO fibres) or short CNT-7 (0.36 x109 WHO fibres)

  Mice: Dose not provided

  Short-term experiment:

  Rat: 2 mg (CNT-7: 0.67 x 109 WHO fibres; short CNT-7: 0.12 x 109 WHO fibres)

  Mice: 0.2 mg of CNT-7 (0.67 x 10^8 WHO fibres)
- *frequency of treatment:* Long-term experiment: single dose
- *control group and treatment*: Positive control: 2 mg crocidolite (6 x 10^9 WHO fibres)
- *historical control data*
- *post exposure observation period*:

  Long-term experiment: sacrificed after 12 months (rat)

  Short-term experiment: sacrificed after 1, 7, 15 and 30 d (rat and mice)
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation*

  1. "CNT-7": L: 7.1 µm (median); D: 75 nm; Fibres > 5 µm: 75%
  Metallic impurities: Fe: 0.35 w%; Co, Ni, Mo: < 0.001 w%

  2. "Short CNT-7": L: 2.8 µm (median); D: 75 nm; Fibres > 5 µm: 14%
  Metallic impurities: Fe: 0.35 w%; Co, Ni, Mo: < 0.001 w%

  3. "CNT-M" (aggregated): L: 0.7 µm (median); D: 11.3 nm; Fibres > 5 µm: <1%
  Metallic impurities: Fe: 0.48 w%; Co: 0.49 w% Ni, Mo: < 0.001 w%

  4. "CNT-T" (tangled): L: 3 µm (median); D: 15 nm; Fibres > 5 µm: n/a

Metallic impurities: n/a

Positive control: crocidolite (L: 3 µm, D: 200 nm, Fibres > 5 µm: 5 w%)

- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size (for studies with aerosols, indicate mass median aerodynamic diameter and geometric standard deviation or give other specifications)*
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs*
- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type:*

  Both, CNT-7 and short CNT-7 induced mesothelioma, the latter to a lesser extent.

First tumours after CNT-7 injection developed mesothelioma after 6 months and the majority of animals developed tumours.

In contrast, only one animal developed mesothelioma 12 months after crocidolite injection.

Administration of mesotheliomagenic CNT-7 and short CNT-7 resulted - like asbestos - in an early and persistent recruitment of monocytic Myeloid Derived Suppressor Cells (M-MDSC) as detected in peritoneal lavage. In contrast, non-carcinogenic CNT-M and CNT-T did only transiently induce M-MDSC. The authors speculated that the immunosuppressive activity of these monocytes towards T-lymphocytes, which was verified by in vitro proliferation tests, is an important step in mesothelioma formation by MWCNT in addition to an early inflammatory neutrophil recruitment.

The specificity of the early M-MDSC accumulation by MWCNT or asbestos was demonstrated by injection of other substances, such as silica and LPS, which only induced an inflammatory but no immunosuppressive response.

Mice did not develop mesothelioma, even after multiple injections of CNT-7 (data not shown). This was attributed to the observation that the monocytic cells harvested from the peritoneum of treated mice did not possess a significant and persistent immunosuppressive activity (no accumulation of M-MDSC).

- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency*
- *statistical methods and results (unless already described with specific test results above)*

2.1.1.6 [Study 6]

Study reference:

Rittinghausen et al. (2014): The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. Particle and Fibre Toxicology 11

Detailed study summary and results:

Test type

Long-term observation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWCNT
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* Rat/Wistar Han rCrl:W1 [Han]/ m
- *No. of animals per sex per dose:* 50/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration :* Intraperitoneal injection
- *duration of test/exposure period*
- *doses/concentration levels, rationale for dose level selection*: exposure of 1 x 10^9 and 5 x 10^9 WHO fibres per animal, respectively
  This corresponds to: 0.2 and 1 mg (MWCNT-A); 6 and 3 mg (MWCNT-B); 0.08 and 0.4 mg (MWCNT C); 0.25 and 1.4 mg (MWCNT D);
  Samples dispersed in surfactant-like 1,2 dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) to prevent aggregation.
- *frequency of treatment:* Single dose exposure
- *control group and treatment:* Positive control: 1x 108 WHO fibres of amosite asbestos
- *historical control data*
- *post exposure observation period*: two-years
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:*

  1. "MWCNT A": LWHO fibres: ~ 8.6 µm; DWHO fibres: ~ 85 nm; No. of fibres > 20 µm: ~ 3.1%
  2. "MWCNT B": LWHO fibres: ~ 9.3 µm; DWHO fibres: ~ 62 nm; No. of fibres > 20 µm: ~ 9.4%
  3. "MWCNT C": LWHO fibres: ~ 10.2 µm; DWHO fibres: ~ 40 nm; No. of fibres > 20 µm: ~ 11.8%
  4. "MWCNT D": LWHO fibres: ~ 7.9 µm; DWHO fibres: ~ 37 nm; No. of fibres > 20 µm: ~ 2.1%
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*

- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size (for studies with aerosols, indicate mass median aerodynamic diameter and geometric standard deviation or give other specifications)*
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs*
- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings:* The test tailor-made MWCNT types exhibited a rigid-fibre like morphology with slight geometrical variations and a considerable share of WHO fibres.
  Immunohistochemical marker staining demonstrated similarity to mesotheliomas induced by asbestos that occurred in humans : 64% of mesotheliomas were of the sarcomatoid type, 32% of the biphasic and 4% of the epitheloid type.
  Most tumours invaded peritoneal organs, the diaphragm in particular

- *tumour incidence data by sex, dose and tumour type:* All types induced malignant mesotheliomas in all dose groups. Though a clear dose-dependency was not observed, mesothelioma development was more rapid and severe in case of more straight MWCNT types compared to more flexible ones and even to amosite asbestos.

- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*

- *tumour latency:* MWCNT D – the most curved type compared to the rather straight types A-C - showed the highest latency in mesothelioma development, indicating that curvature may be an additional factor of fibre geometry for carcinogenic potency of MWCNT.

- *statistical methods and results (unless already described with specific test results above)*

2.1.1.7 [Study 7]

Study reference:

Nagai et al. (2011)*:* Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. Proc Natl Acad Sci U S A 108 (49); E1330-8.

Detailed study summary and results:

Test type

Long-term observation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7 (Mitsui); Needle-like fibres of high crystallinity
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* Rat/Fisher 344/ f and Rat/Brown Norway F1 hybrid/ m
- *No. of animals per sex per dose:* n=11-60/ group
- *Age and weight at the study initiation*: no data

Administration/exposure

- *Route of administration :* Intraperitoneal injection
- *duration of test/exposure period:* Two administrations in in a 1-week interval
- *doses/concentration levels, rationale for dose level selection:* 1 or 10 mg/rat NT 50a, NTtngl or NT145, 10 mg/ rat NT50b
  In addition a non- agglomerated subfraction of NT50a with fibre numbers equivalent to 1 mg NT145 was tested. [= eq. NT50a(-agg*)]

- *frequency of treatment*
- *control group and treatment:* no positive control
- *historical control data*
- *post exposure observation period:* 1 year
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:*

  1. "NT50a" = MWNT-7 (Mitsui); Needle-like fibres of high crystallinity, highly aggregated D: 49.95 nm, L: 5.3 µm
  2. NT50a(-agg*)] subfraction of NT50a
  3. „NT50b"(Showa Denko); Fibres of high crystallinity, highly aggregated; D: 52.4 nm, L: 4.6 µm
  4. "NT145" (Showa Denko); "thick" nanotubes, aggregation low; D: 143.5 nm; L: ~4.3 µm
  5. "NTtngl" (Sowa Danko); Tangled nanotubes, very high aggregation.; D: 15 nm; L: 3 µm

- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size (for studies with aerosols, indicate mass median aerodynamic diameter and geometric standard deviation or give other specifications)*
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death:* It is noted that early half of the rats administered 10 mg i.p. of either MWCNT type died within several days and the remaining animals were used as as the 10 mg-injection group. The mechanism for acute death is unknown, though arterial and/or lymphatic embolism is suspected (Nagai et al., 2013).
- *clinical signs*

- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type*: 1 mg of crystalline NT50a or an equivalent amounts (eq.) of NT50a(-agg*) induced malignant mesothelioma with a higher frequency and earlier progression than 1 mg of NT145. (based on survival rates).
  Incidences of 10 mg NT145 or of NT50b were as high as of 1 mg. NT50a or eq. NT50a(-agg*). 10 mg of NTtngl did not induce mesotheliomas.
  86% of mesotheliomas exhibited a sarcomatoid histology (0.9% had an epithelioid, 12.1% a biphasic phenotype).
  MWCNT-induced mesotheliomas shared homozygous deletion of Cdkn2a/2b tumour suppressor genes, similar to asbestos-induced mesotheliomas.

- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *tumour incidence data by sex, dose and tumour type*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency*
- *statistical methods and results (unless already described with specific test results above)*

2.1.1.8 [Study 8]

Study reference:

Nagai et al. (2011)*:* Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. Proc Natl Acad Sci U S A 108 (49); E1330-8.

Detailed study summary and results:

Test type

Short-term observation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* Rat/Fisher 344, f and Rat/Brown Norway F1 hybrid, m
- *No. of animals per sex per dose:* no data
- *Age and weight at the study initiation*: no data

Administration/exposure

- *Route of administration – oral (gavage, drinking water, feed), dermal, inhalation (aerosol, vapour, gas, particulate), other*
- *duration of test/exposure period*
- *doses/concentration levels, rationale for dose level selection:* 1 mg/rat NT 50a 1 or 5 mg NT145 or NTtngl
- *frequency of treatment:* Single exposure
- *control group and treatment:* no positive control
- *historical control data*
- *post exposure observation period:* 1 month
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:*

  1. "NT50a" = MWNT-7 (Mitsui); Needle-like fibres of high crystallinity, highly aggregated
  D: ~50 nm, L: 5.3 µm
  2. "NT145" (Showa Denko); "thick" nanotubes, aggregation low;D: 143.5 nm; L: ~4.3 µm
  3. "NTtngl" (Sowa Danko); Tangled nanotubes, very high aggregation; D: 15 nm; L: 3 µm
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size (for studies with aerosols, indicate mass median aerodynamic diameter and geometric standard deviation or give other specifications)*
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs:* The needle-like NT50a was a potent inducer of fibrotic inflammation, causing severe fibrotic peritonitis and mesothelial proliferation.

  Thicker or tangled MWCNT (NT145 and NTtngl, respectively) induced mild inflammatory fibrosis but no mesothelial proliferation. All tested MWCNT types were phagocytosed by macrophages and caused local granuloma formation.
- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type*
- *local or multi-site responses*
- *progression of lesions to malignancy*

- *gender and/or species-specific responses*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency:* Concomitant in vitro cytotoxicity studies suggested that the difference in the extent of inflammation between the MWCNT types was related to the ability to induce direct mesothelial injury at the end of the one month observation time.
- *statistical methods and results (unless already described with specific test results above)*

2.1.1.9 [Study 9]

Study reference:

Sargent et al. (2014): Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes. Particle and Fibre Toxicology 11.

Detailed study summary and results:

Test type

Short-term tumour promotion study

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities:* Trace metal contamination 1.32% (Fe: 1.06%)
- *Batch number:* Mitsui-7, Hodogaya, lot # 061220-31

Test animals

- *Species/strain/sex:* Mouse/B6C3F1 hybrid, m
- *No. of animals per sex per dose:* no data
- *Age and weight at the study initiation*: no data

Administration/exposure

- *Route of administration* – inhalation (whole body)

- *duration of test/exposure period*: Challenge: 5 hours/ day; Controls: filtered air for 15 days
- *doses/concentration levels, rationale for dose level selection:*
  1. Initiation: methylcholanthrene; 10 µg/g bw
  2. Challenge: MWNT-7; 5 mg/m^3
- *frequency of treatment:* Two step treatment*:*
  1. Initiation: Single i.p. dose of MCA or vehicle
  2. Challenge (1 week after initiation): MWNT-7; 5 hours/ day or filtered air (controls) for 15 days.
- *control group and treatment*
- *historical control data*
- *post exposure observation period:* 17 month
- *vehicle:* corn oil
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:*
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure :* whole body
- *analytical verification of test atmosphere concentrations*
- *particle size:* MWNT-7: MMAD: 1.59 µm, CMAD: 0.42 µm
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs:* strong promoter of pulmonary adenomas

focal adenomatous hyperplasia, macrophage infiltrations into the lung as well as lesion-associated foreign material in the alveolar tissue, the interstitium, and also within alveolar macrophages. Incidences for the (primary) hyperplasia were 27% for MCA + MWCNT; compared to 5% MWCNT alone and 2% each for MCA and air control.

- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type*
- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *tumour incidence data by sex, dose and tumour type:* 90.5% of MWCNT-exposed mice developed one or the other tumour when pre-treated with MCA, compared to 14% without pre-treatment, which was close to the air-control group (13%).
  Several pre-treated mice also developed malignant serosal tumours consistent with sarcomatous mesothelioma, as demonstrated by podoplanin immunostaining.

- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose:* The particle lung burden in MWCNT-exposed mice was equivalent to occupational settings (31.2 µg/lung).

- *tumour latency:* strong promoter of adenocarcinomas in mice; The significantly increased incidence of focal adenomatous alveolar hyperplasia after 15 days of inhalation exposure by MWNT-7 w/o MCA compared to air-exposed controls indicated an initiator role of MWNT-7 in carcinoma development in mice.

- *statistical methods and results (unless already described with specific test results above)*

2.1.1.10 [Study 10]

Study reference:

Takagi et al. (2008) : Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube. Journal of Toxicological Sciences 33 (1); 105-116

Detailed study summary and results:

Test type

Medium-term observation

No standard test guideline

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7 (Mitsui)
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities:* 0.5% methyl cellulose
- *Batch number*

Test animals

- *Species/strain/sex:* Mouse/SLC mutants/m (p53+/-heterozygosity)
- *No. of animals per sex per dose:* 19/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration:* Intraperitoneal injection
- *duration of test/exposure period*
- *doses/concentration levels, rationale for dose level selection: 3 mg/mouse (1×10^9 MWCNT)*
- *frequency of treatment:* single injection
- *control group and treatment:* Positive control: crocidolite (3 mg/mouse)
- *historical control data*
- *post exposure observation period:* 25 weeks (due to 100% mortality)
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:* Aggregates among dispersed rod-shaped or fibrous particles
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*

- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size (for studies with aerosols, indicate mass median aerodynamic diameter and geometric standard deviation or give other specifications)*
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death:* All MWCNT-treated animals died before the end of the observation period.
- *(indicate number died per sex per dose and time to death)*
- *clinical signs:* MWCNTs induced mesothelioma, which were invasive to the abdominal wall, diaphragm, liver parenchyma and pancreas, and in some case involving the thoracic cavity. Distant metastasis was not observed (day 172 after injection).
- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type*
- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *tumour incidence data by sex, dose and tumour type*

- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency:* Large tumours invaded the abdominal wall, diaphragm, liver parenchyma, and pancreas, rarely also involving the thoracic cavity. Distant metastases were not observed. Fibre-laden cells were not only found in peritoneal lesions but also in the liver and in mesenteric lymph nodes. The overall mesothelioma incidence at day 84 post i.p. treatment was even higher for MWCNT (87.5%) compared to Crocidolite (77.8%).
- *statistical methods and results (unless already described with specific test results above)*

2.1.1.11 [Study 11]

Study reference:

Takagi et al. (2012): Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice. Cancer Science 103 (8); 1440-1444.

Detailed study summary and results:

Test type

Long-term observation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7 (Mitsui)
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* mouse/ SLC mutants/ m (p53+/-heterozygosity)
- *No. of animals per sex per dose:* 20/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration:* Intraperitoneal injection

- *duration of test/exposure period:*
- *doses/concentration levels, rationale for dose level selection:* 3, 30, 300 µg/mouse, corresponding to 1x106, 1x107, 1x108 particles/mouse
- *frequency of treatment:* Single injection
- *control group and treatment:* no positive control
- *historical control data*
- *post exposure observation period*: up to 1 year
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:* MWNT-7; D: 100 nm; L: 27.5% > 5 µm (100% < 20 µm)Fe 0.35%
  Aggregates among dispersed rod-shaped or fibrous particles in administered suspension (0.5% methyl cellulose).
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test*
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size (for studies with aerosols, indicate mass median aerodynamic diameter and geometric standard deviation or give other specifications)*
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death:* Dose-dependent mesothelioma induction with cumulative incidence of 5/20, 17/20 and 19/20, respectively (increasing particle numbers). Most mesothelioma were lethal.
- *clinical signs:* The 15 surviving mice at low dose treatment showed focal mesothelial atypical hyperplasia. No mesothelioma was observed in the vehicle control group.

- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings*: Histology of the mesotheliomas ranged from a differentiated epithelioid type to an undifferentiated sarcomatous type. Osteoid and rhabdoid differentiations, both known in human asbestos mesothelioma cases, were found in nine mice (across all dose groups). Peritoneal fibrosis, peritoneal adhesion and formation of foreign body granulomas towards agglomerated MWCNT were dose dependent and minimal in the low-dose group.
- *tumour incidence data by sex, dose and tumour type:*
- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency:* Severity of peritoneal adhesion and granuloma formation was dose-dependent, as estimated by logarithmic approximation of mesothelioma mortality plots. However, the time of tumour onset was apparently independent of the dose, which let the authors favour an indirect carcinogenic effect by humoral stimuli on mesthelial cells near loci of frustrated pahagocytosis.
- *statistical methods and results (unless already described with specific test results above)*

2.1.1.12 [Study 12]

Study reference:

Sakamoto et al. (2009): Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. Journal of Toxicological Sciences 34 (1); 65-76.

Detailed study summary and results:

Test type

Long-term observation

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7 (Mitsui)

- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities:* Agglomerates and dispersed as multi-sized rod-shaped or fibrous particles in administered suspension (2% carboxymethyl cellulose)
- *Batch number*

Test animals

- *Species/strain/sex:* Rat/Fischer 344 DuCrlCrlj/ m
- *No. of animals per sex per dose:* n=7/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *Route of administration:* Intrascrotal injection
- *duration of test/exposure period:* Single injection
- *doses/concentration levels, rationale for dose level selection*
- *frequency of treatment:*
- *control group and treatment:* Positive control: crocidolite (2 mg/kg bw)
- *historical control data*
- *post exposure observation period:* 52 week
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:* MWNT-7; D : 82% in range 70-110 nm; L : 72.5% in range 1-4 μm Fe 0.35% Agglomerates and dispersed as multi-sized rod-shaped or fibrous particles in administered suspension (2% carboxymethyl cellulose)
- *actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test:* 1 mg/kg bw
- *substance concentration (ppm) to the actual dose, if applicable*
- *satellite groups and reasons they were added*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure ("whole body", "oro-nasal", or "head only"), exposure data*
- *analytical verification of test atmosphere concentrations*
- *particle size (for studies with aerosols, indicate mass median aerodynamic diameter and geometric standard deviation or give other specifications)*

- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- *mortality and time to death (indicate number died per sex per dose and time to death)*
- *clinical signs:* Rats treated with 2 mg/kg bw crocidolite also developed granulomas but no mesotheliomas
- *body weight gain*
- *food/water consumption*
- *ophthalmoscopic examination*
- *clinical chemistry*
- *haematology*
- *urinalysis*
- *organ weights*
- *necropsy findings: nature and severity*
- *histopathological findings: nature and severity*
- *tumour incidence data by sex, dose and tumour type*
- *local or multi-site responses*
- *progression of lesions to malignancy*
- *gender and/or species-specific responses*
- *tumour incidence data by sex, dose and tumour type*
- *mode of action (genotoxic, non-genotoxic)*
- *toxic response data by sex and dose*
- *tumour latency:* MWCNTs induced mesotheliomas in 6 of 7 treated rats that died prior to the end of the study. The overall indicence of mesothelioma was even significantly higher than those of crocidolite-treated rats.
  These advanced stage tumours, which developed from mesothelial hyperplasia over polyploidy or papillary mesotheliomas, invaded into adjacent tissues and organs and metastasized into the pleura.
  Beside these mesothelial proliferative lesions, granulomas with high cellularity, including macrophages and multinucleated giant cells were observed.

- *statistical methods and results (unless already described with specific test results above)*

2.2 Specific target organ toxicity – repeated exposure

2.2.1 Animal data

2.2.1.1 [Study 1]

Study reference:

Kasai et al. (2015) Thirteen-week study of toxicity of fiber-like multi-walled carbon nanotubes with whole-body inhalation exposure in rats. Nanotoxicology 1-10.

Detailed study summary and results:

Test type

Subchronic study (90d)

Followed OECD TG 413

Test substance *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7

- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity:* > 99.5%
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number:* Hodogaya, Lot No. 071223, 080126

Test animals

- *Species/strain/sex:* Rat/F344 DuCrlCrlj/ m/f
- *No. of animals per sex per dose:* 10/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *route of administration*: inhalation (dry aerosol)
- *duration and frequency of test/exposure period:* 6 h/day, 5 days/week for 13 weeks
- *doses/concentration levels, rationale for dose level selection:* 0, 0.2, 1 or 5 mg/m^3 MWCNT aerosol MWCNT mass doses corresponded to number concentrations of about 115,000, 577,0000, and 2,933,000 cpm
- *post exposure observation period*

- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *control group and treatment*
- *test substance formulation/diet preparation, achieved concentration by sex and dose level, stability and homogeneity of the preparation*
- *actual dose (mg/kg bw/day) and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable*
- *statistical methods*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure:* whole body
- *analytical verification of test atmosphere concentrations*
- *particle size:* MWNT-7: D: 90.7 nm (mean); L: 5.7 µm (mean; 48.7% > 5 µm); MMAD = 1.4-1.6 µm (~80% mass as inhalable fraction)
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings and toxic response/effects by sex and dose level. If no effects occurred, explicitly note "No effects".

- *body weight and body weight changes*
- *food/water consumption*
- *description, severity, time of onset and duration of clinical signs (reversible, irreversible, immediate, delayed):* The lung burden of males at the highest exposure concentration was estimated as 120 µg, that one of females was about 80 µg left lung (accounting for the lower respiratory frequency of female rats). Accordingly, 13.7 µg per left lung resulted in granulomatous changes in 40% of the animals after an additional 4 week recovery period, granulomatous changes were observed in 4/5 rats). ~2.3 µg/left lung did not cause granulomatous changes.
- *sensory activity, grip strength and motor activity assessments (when available)*
- *ophthalmologic findings: incidence and severity*
- *haematological findings: incidence and severity*
- *clinical biochemistry findings:* Concentration-dependent increase in inflammatory BAL parameters (PMN and lymphocyte numbers, LDH, ALP and TP) in both sexes from 0.2 mg/m3 and above.
- *gross pathology findings:* Lung weights: significantly increased at 1 and 5 mg/m^3

Increased numbers of neutrophils and lymphocytes in the lungs, and hyperplasia of goblet cells in the nasal cavity and nasopharynx were observed.

Multifocal fibrosis of the alveolar wall was observed at 1 mg/m³ and above in both sexes. The incidence and severity of fibrosis and granuloma formation increased at higher exposure concentrations.

Inflammatory infiltration in the visceral pleural and subpleural areas was induced at 5 mg/m3.
MWCNT were primarily found within alveolar macrophages (longer fibres only partially phagocytosed in AM with foamy cytoplasm), but few single MWCNT were also found in bronchiolar and alveolar spaces. Occasionally, single MWCNTs were detected in the visceral subpleural areas and in the parietal pleura at the diaphragm.

- *histopathology findings:* Inflammation-related histopathologic lesions included foreign body granulomas with increased numbers of associated multinucleated MWCNT-laden macrophages in the lung at 1 and 5 mg/m3 in females and males and even at 0.2 mg/m3 in males.

- *mortality and time to death (if occurring)*

2.2.1.2 [Study 2]

Study reference:

Umeda et al. (2013)*:* Two-week Toxicity of Multi-walled Carbon Nanotubes by Whole-body Inhalation Exposure in Rats. J Toxicol Pathol 26 (2); 131-40.

Detailed study summary and results:

Test type

Subacute study (14d)

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity:* 99.8%
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number:* Hodogaya, Lot No. 080126

Test animals

- *Species/strain/sex:* Rat/F344 DuCrlCrlj rats, m/f
- *No. of animals per sex per dose:* 10/group
- *Age and weight at the study initiation*: no data

Administration/exposure

- *route of administration*: inhalation (aerosol)
- *duration and frequency of test/exposure period:* 14d/ 6 h/day, 5 days/week
- *doses/concentration levels, rationale for dose level selection*: 0, 0.2, 1 or 5 mg/m^3
- *post exposure observation period*: 4-weeks
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *control group and treatment*
- *test substance formulation/diet preparation, achieved concentration by sex and dose level, stability and homogeneity of the preparation*
- *actual dose (mg/kg bw/day) and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable*
- *statistical methods*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure:* whole body
- *analytical verification of test atmosphere concentrations*
- *particle size:* D: 88 nm (mean); L: 5.0 µm (mean, 38.9% > 5 µm)
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings and toxic response/effects by sex and dose level. If no effects occurred, explicitly note "No effects".

- *body weight and body weight changes*
- *food/water consumption*
- *description, severity, time of onset and duration of clinical signs (reversible, irreversible, immediate, delayed)*
- *sensory activity, grip strength and motor activity assessments (when available)*

- *ophthalmologic findings: incidence and severity*
- *haematological findings: incidence and severity*
- *clinical biochemistry findings: incidence and severity*
- *gross pathology findings:* Persistent deposition of MWCNTs in the lungs of all MWCNT-exposed groups was observed. MWCNT deposited in bronchus-associated lymphoid tissue (BALT) and peritracheal lymph nodes were found after exposure and at 1 and 5 mg/m3 after p.e.
  Numbers of neutrophils and lymphocytes in BALF tended to remain elevated in the 5 mg/m3 dose group, the numbers of multinucleated macrophages even increased at 5 mg/m3 after post-exposure. Albumin and TP were still elevated.in the 1 and 5 mg/m3 groups, ALP in the 5 mg/m^3.
  The total amounts of MWCNTs in the 5 mg/m^3 group was approximately 43.4 µg/lung at the end of the 2-week exposure period and approximately 41.2 µg/lung at the end of the 4-week p.e. period.

- *histopathology findings:* In histopathology, granulomatous changes and slight alveolar fibrosis occurred in the lung at the highest dose. Granulomatous changes slightly increased at the end of the p.e.
  At 1 and 5 mg/m^3, goblet cell hyperplasia in the nasal cavity and nasopharynx were observed, which largely regressed at the end of p.e.

  A NOAEC of 0.2 mg/m^3 was derived by the study authors based on BAL parameters and histopathologic findings

- *mortality and time to death (if occurring)*

2.2.1.3 [Study 3]

Study reference:

Rydman et al. (2014) Inhalation of rod-like carbon nanotubes causes unconventional allergic airway inflammation. Part Fibre Toxicol 11; 48.

Detailed study summary and results:

Test type

Short-term study (4d)

No standard test guideline followed

lung only organ investigated

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier*: 1. "rCNT": XNRI MWNT-7 (Mitsui)
  2. "tCNT" MWCNTs 8-15 nm (CheapTubes)
- *EC number (if different from the substance identified in the CLH dossier)*

- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity:* 1. 99.79% (rigid, rod-like structure)
  2. 99.76% (flexible tangled structure)
- *Impurities (or a note that the impurities do not affect the classification)*
- *Batch number*

Test animals

- *Species/strain/sex:* Mouse: C57BL/6, BALB/c/ f and KitWsh/HNihrJaeBsmJ (mast-cell deficient)/ f
- *No. of animals per sex per dose:* 10-20 (7-9)/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *route of administration* – inhalation
- *duration and frequency of test/exposure period:* 4 days (4h/d)
- *doses/concentration levels, rationale for dose level selection:* 6.2-8.2 mg/m^3 for rCNT and 17.5-18.5 mg/m^3 for tCNT
- *post exposure observation period:* Mice were sacrificed immediately or 24 h after last exposure
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *control group and treatment*
- *test substance formulation/diet preparation, achieved concentration by sex and dose level, stability and homogeneity of the preparation*
- *actual dose (mg/kg bw/day) and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable*
- *statistical methods*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure*: whole body
- *analytical verification of test atmosphere concentrations*
- *particle size:* 1. D: > 50 nm; L: ~13 µm
  2. D: 8-15 nm; L: 10-50 nm
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings and toxic response/effects by sex and dose level. If no effects occurred, explicitly note "No effects".

- *body weight and body weight changes*
- *food/water consumption*
- *description, severity, time of onset and duration of clinical signs (reversible, irreversible, immediate, delayed):* Evidence was presented that inhaled fibre-like MWCNT (rCNT) can induce immunity-mediated allergic-like airway inflammation in healthy mice, including effects such as marked eosinophilia accompanied by mucus hypersecretion, airway hyper responsiveness, and the expression of Th2-type cytokines and eosinophil chemoattractants.
- *sensory activity, grip strength and motor activity assessments (when available)*
- *ophthalmologic findings: incidence and severity*
- *haematological findings: incidence and severity*
- *clinical biochemistry findings: incidence and severity*
- *gross pathology findings:* Both tests induced the recruitment of inflammatory cells, especially eosinophils. In the lung, rCNT fibres were detected in alveolar macrophages in interstitial areas. Macrophages were found to undergo “frustrated phagocytosis” and form foreign-body giant cells.
- *histopathology findings: incidence and severity*
- *mortality and time to death (if occurring)*

2.2.1.4 [Study 4]

Study reference:

Porter et al. (2013) Acute pulmonary dose-responses to inhaled multi-walled carbon nanotubes. Nanotoxicology 7 (7); 1179-1194

Detailed study summary and results:

Test type

Short-term study (12d)

No standard test guideline followed

examination limited to the respiratory tract; histopathology included nose, lung and tracheobronchial lymph nodes

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*

- *Degree of purity:* no data
- *Impurities:* 1.32% metal contamination (1.06% iron)
- *Batch number*: Hodogaya, Lot No. 061220-31

Test animals

- *Species/strain/sex:* Mouse/C57BL/6J/ m
- *No. of animals per sex per dose:* 7-9/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *route of administration* – inhalation
- *duration and frequency of test/exposure period*: exposure for 2, 4, 8, or 12 days
- *doses/concentration levels, rationale for dose level selection*: 0 or 10 mg/m^3
- *post exposure observation period*
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *control group and treatment*
- *test substance formulation/diet preparation, achieved concentration by sex and dose level, stability and homogeneity of the preparation*
- *actual dose (mg/kg bw/day) and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable*
- *statistical methods*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure:* whole body
- *analytical verification of test atmosphere concentrations*
- *particle size:* Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode): MMAD = 1.5 µm
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*
- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings and toxic response/effects by sex and dose level. If no effects occurred, explicitly note "No effects".

- *body weight and body weight changes*
- *food/water consumption*
- *description, severity, time of onset and duration of clinical signs (reversible, irreversible, immediate, delayed)*
- *sensory activity, grip strength and motor activity assessments (when available)*
- *ophthalmologic findings: incidence and severity*
- *haematological findings: incidence and severity*
- *clinical biochemistry findings: incidence and severity*
- *gross pathology findings:* Dose-dependent increase in whole lung lavage (WLL) markers for lung inflammation (PMN), lung cytotoxicity (LDH) and alveolar air-blood barrier integrity (albumin) over controls.

- *Lung histopathology revealed:* A linear increase in lung burden ranging from 6.6 and 30.6 µg after 2 and 12 days, respectively, which the authors deemed occupationally relevant equivalent doses

- *histopathology findings:* Lung histopathology revealed bronchiolocentric inflammation, bronchiolar epithelial hyperplasia and hypertrophy, minimum to mild bronchiolocentric fibrosis, vascular changes (medial hypertrophy and contraction, mural neutrophil infiltrates and rare mural MWCNT) and rare pleural penetration.
  Translocation of MWCNT to the lymph nodes was observed, accompanied by lymph node enlargement with paracortical hyperplasia (the authors speculated that these lymph nodes are a major site of lung clearance and that activated macrophages containing MWCNT that reach the pleura travel through these lymphatics).

- *mortality and time to death (if occurring)*

2.2.1.5 [Study 5]

Study reference:

Mercer et al. (2013)*:* Distribution and fibrotic response following inhalation exposure to multi-walled carbon nanotubes. Particle and Fibre Toxicology 10

Detailed study summary and results:

Test type

Short-term study (12d)

No standard test guideline followed

Test substance

- *Indicate if the test material used in the study is equivalent to the substance identified in the CLH dossier:* MWNT-7
- *EC number (if different from the substance identified in the CLH dossier)*
- *CAS number (if different from the substance identified in the CLH dossier)*
- *Degree of purity*
- *Impurities:* 1.32% metal contamination (1.06% iron)
- *Batch number:* Hodogaya, Lot No. 061220-31

Test animals

- *Species/strain/sex:* Mouse/C57BL/6J/ m
- *No. of animals per sex per dose:* 7-9/group
- *Age and weight at the study initiation:* no data

Administration/exposure

- *route of administration* – inhalation (Stable, acoustical-based generated aerosol)
- *duration and frequency of test/exposure period:* 12 days
- *doses/concentration levels, rationale for dose level selection:* 0 or 5 mg/m^3
- *post exposure observation period:* Post- observation time points: 1, 14, 84, 168, and 336 days
- *vehicle: identification, concentration and volume used, justification of choice of vehicle (if other than water)*
- *control group and treatment*
- *test substance formulation/diet preparation, achieved concentration by sex and dose level, stability and homogeneity of the preparation*
- *actual dose (mg/kg bw/day) and conversion factor from diet/drinking water test substance concentration (ppm) to the actual dose, if applicable*
- *statistical methods*

For inhalation studies:

- *type of inhalation exposure and test conditions (e.g.: exposure apparatus)*
- *method of exposure:* whole body
- *analytical verification of test atmosphere concentrations*
- *particle size:* L: 4.3 µm (mean); Aerodynamic diameter = 1.3 µm (mass mode), 0.42 µm (count mode):MMAD = 1.5 µm.
- *type or preparation of particles (for studies with aerosols)*

For dermal studies:

- *area covered (e.g. 10% of body surface)*
- *occlusion (e.g. semi-occlusive)*
- *total volume applied*

- *removal of test substance (e.g. water or solvent)*

Results and discussion

Describe the relevant findings and toxic response/effects by sex and dose level. If no effects occurred, explicitly note "No effects".

- *body weight and body weight changes*
- *food/water consumption*
- *description, severity, time of onset and duration of clinical signs (reversible, irreversible, immediate, delayed)*
- *sensory activity, grip strength and motor activity assessments (when available)*
- *ophthalmologic findings: incidence and severity*
- *haematological findings: incidence and severity*
- *clinical biochemistry findings: incidence and severity*
- *gross pathology findings:* Progressive alveolar fibrosis was observed and persisted over the whole postexposure period (increase of connective tissue thickness in the alveolar region by 70% 336 days after exposure).
  This was in line with measurements of inflammatory BAL parameters (PMN, LDH, and albumin), which increased rapidly (day 1) and declined slowly over postexposure time (still significantly increased on day 168). Smaller MWCNT structures were rapidly incorporated into the alveolar interstitium.
  The initial p.e. lung burden was 21.8 µg, 84% of which deposited in the alveolar region, mainly in alveolar macrophages (56%). Clearance reduced the alveolar macrophage burden of MWCNT by 35 percent between 1 and 168 days p.e., while the content of MWCNTs in the alveolar tissue increased by 63 percent.
- *histopathology findings: incidence and severity*
- *mortality and time to death (if occurring*